IN VITRO MORPHOGENIC STUDIES IN *DIANTHUS* AND ASSESSMENT OF CLONING FIDELITY

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INTRODUCTION

Dianthus is one of the world's most popular, economic and important cut flowers due to perpetual flowering and presence of new single- and multi-colour cultivars. It belongs to family Caryophyllaceae and is native of the Mediterranean region.

The genus *Dianthus* consists of more than 300 species including the ornamentals such as Carnations (*D. caryophyllus* L.), Pinks (*D. chinensis*) and Sweet Williams (*D. barbatus*). *D. caryophyllus* is a top-selling ornamental crop worldwide. *D. chinensis* is short-lived, perennial garden plants, native to China and northern parts of India, blooming in several colours. Certain varieties of pinks have a strong clove scent, which has made them popular in perfumes for more than 2,000 years. It has a wide range of medicinal uses as well. *D. barbatus* (biennial carnation) is native to the mountains of southern Europe from the Pyrenees east to the Carpathians and the Balkans, with a variety disjunct in north-eastern China, Korea, and south-easternmost Russia. Its traditional use is in landscaping and cut flowers.

Dianthus is generally short, therefore they can be rapidly swamped by weeds which compete with the plants and can cause production problems. Commercial growers cultivate them in greenhouses using sterile soil which greatly improves weed and disease management (Galbally and Galbally, 1997). Thrips, aphids, mites, *Helicoverpa* caterpillars, and other arthropod pests are also serious threat to the plant which can generally be controlled by chemical treatment (Bird, 1994). They are also routinely affected by various bacterial and fungal pathogens. Cucumber mosaic virus (serologically closely related to strains CMV-C and CMV-D) was reported for the first time as a natural infection of *D. barbatus* causing leaf crinkle and stunting of plants by Aminuddin *et al.* (2007).

Certain abiotic stresses like low temperature (<10°C), low nitrogen, high ammoniacal nitrogen or low boron levels enhance physical abnormalities like calyx-splitting and slabside. Carnation breeders constantly seek new varieties with improved horticultural traits such as disease and pest resistance and long vase-life. Due to highly heterozygous nature, the carnation does not come true to its parent when grown from seed. Consequently, under horticultural conditions, this method of propagation is only used in selection of new varieties although annual carnations tend

to be propagated by seeds. Furthermore, carnations can also be propagated by tissue culture techniques. Genetic engineering based on tissue culture technology provides an alternative way to overcome these problems.

In vitro technology has often been used as a promising avenue to plant improvement for research and practical applications. It can facilitate inter-specific hybridisation and genetic manipulations through molecular breeding approaches. It serves as a means of clonal propagation of economically important species and as an adjunct to traditional means of plant modification as it increases the mutation rate of plant cells, resulting in somaclonal variation, which significantly affects plant improvement (Sajid and Pervaiz, 2008).

Zooming into the various aspects of plant tissue culture it can be stated that growth and morphogenesis of plant tissue under *in vitro* conditions are largely governed by the composition of the culture medium. Different culture media formulation proposed consist of several basal components which includes mineral nutrients, vitamins, amino acids, growth regulators, sugars, agar and water in various concentrations.

The mineral salts in media can be broadly classified into two groups: microelements and macroelements. Microelements including Fe, Cu, Zn, Co, Mo, B, Cl and I are required in relatively low concentration but their deficiency can have catastrophic effects. Mg, Ca, S, P, K and N are considered as macroelements since they are required in high concentration. Vitamins, amino acids, growth regulators and sugars act as organic supplements in the medium. Agar and Phytagel are added as gelling agents. All these components fulfil the one or more requirements of plant under *in vitro* conditions.

Scanning of the plant tissue culture literature leads to the conclusion that MS is the most frequently used medium. The MS basal medium proposed by Murashige and Skoog (1962) was originally developed for the culture of tobacco pith callus. Many different media are basically MS with minor changes.

Several heavy metals play important roles in the regeneration of plant tissue cultures as microelements. Silver as silver nitrate ($AgNO_3$) and cobalt as cobalt chloride ($CoCl_2$) act as ethylene inhibitor. Copper (Cu) and nickel (Ni) proved to be effective in stimulating morphogenesis, shoot regeneration and callus induction**.**

Tissue culture regenerated plants have new mutations which occur less frequently in field situations known as somaclonal variations. Tissue culture regenerated somaclones exhibit more than 90% survival in the field (Evans *et al*., 1981). Moreover, tissue culture serves to maintain the clonal fidelity of the germplasm when used as a tool for germplasm conservation. Clonally true to type propagation of plants by a variety of tissue and cell culture methods better known as micropropagation, is now the most commercially efficient and practically oriented plant biotechnology (Sajid and Pervaiz, 2008).

Variation is understood to be generated via a combination of genetic and/or epigenetic changes. A lack of any phenotypic variation between regenerants does not necessarily imply a lack of genetic (or epigenetic) change, and it is therefore of interest to assay the outcomes of tissue culture at the genotypic level. These *in vitro* raised culture variants can be characterized at molecular level. Changes in genome organization can be revealed by a rapid and unspecific DNA fingerprinting method like the Random Amplified Polymorphic DNA (RAPD) analysis which is a simple method to characterize genetic diversity and clonal fidelity without the need of detailed sequence information about the genomes of interest.

RAPD analysis is a quick and economic technique to identify genetic similarity among genotypes. The pattern of amplified bands obtained by RAPD could be used for clonal fidelity. RAPD based approach is capable of describing the qualitative and quantitative characteristics of the tissue culture induced variation.

Considering these facts, the present work on "*In Vitro* Morphogenic Studies in *Dianthus* and Assessment of Clonal Fidelity" is planned with the following objectives:

1. Standardization of an efficient and reproducible micropropagation protocol for *Dianthus*.
2. To study the effect of heavy metals on *in vitro* shoot morphogenesis.
3. Acclimatization and hardening of regenerated plantlets.
4. Field transfer of regenerated plantlets.
5. Evaluation of clonal fidelity of micropropagated plantlets using RAPD analysis.

REVIEW OF LITERATURE

Carnation is one of the most important cut flower crops grown worldwide on a commercial scale, and ranks the top of cut flowers (Duhoky *et al*., 2009). High commercial value together with continuous consumer demand for new varieties act as the driving force for carnation breeding (Moyal-Ben Zvi and Vainstein, 2007). This literature survey presents a compilation of the *in vitro* propagation and contemporary information on biotechnological advances made in *Dianthus* and molecular analysis using RAPD technique.

In vitro propagation can be achieved by organogenesis and somatic embryogenesis. Organogenesis relies on the production of organs, either directly from an explant or from a callus culture. Somatic embryogenesis relies on plant regeneration through a process analogous to zygotic embryos germination which may be directly or indirectly via callus formation.

Adventitious shoot regeneration in carnation has been reported from various explants such as shoot tips (Baker and Philips, 1962; Earle and Langhans, 1975; Jethwani *et al*., 1994) hypocotyls (Petru and Landa, 1974), leaf (Van Altvorst *et al*., 1992, Kantia and Kothari, 2002), stem segments (Van Altvorst and Koehorst, 1995), axillary buds (Van Altvorst and Koehorst, 1995; Ghosh, 1987), petals (Leshem *et al*., 1988; Nugent *et al*., 1991; Van Altvorst *et al.*, 1992; Messeguer *et al*., 1993; Fisher et al., 1993; Sankhla *et al*., 1994; Nakano *et al*., 1994), anthers (Villalobos, 1981), cotyledons (Nontaswatsri and Fukai, 2005) and ovules (Demmink *et al*., 1987). Shoot organogenesis via callus has been reported from leaf explant of *D. chinensis* by Jethwani and Kothari (1996). Callus induction and plant regeneration has also been reported for *D. chinensis* and *D. barbatus* using anther culture by Nontaswastri *et al*. (2009).

Sankhla *et al.* (1994) reported direct *in vitro* production of flowering shoots from petal explants which upon subculture produced additional adventitious flowering shoots. Direct shoot proliferation without any intervening callus was successfully achieved from shoot tips (Hussain *et al*., 1994; Kallack *et al*., 1996; Pareek *et al.*, 2004; Ali et al., 2008; Duhoky *et al*., 2009), nodal explants (Ali *et al*., 2008; Van Altvorst and Koehorst, 1995; Illahi *et al*., 1995; Pareek *et al*., 2004; Duhoky *et al*. 2009) and cotyledonary nodes (Jethwani and Kothari, 1993). Crouch and Van Staden, (1993) reported shoot tip culture of *D. zeyheri* via cell suspension.

Somatic embryogenesis shows several advantages as compared to other *in vitro* propagation systems, including its high multiplication rates and possibility of cryopreservation of embryogenic callus (Karami, 2008).

Development of somatic embryos by culture of small white callus and directly on the leaf surface has also been observed (Nakano and Mii, 1993a). Indirect somatic embryogenesis has been reported from stem tissues in cell suspension culture by Choudhary and Chin (1995). Root explants excised from carnation plants maintained *in vitro* formed somatic embryos via friable callus formation which were subsequently grown to mature plantlets indicating root explants are also competent enough for somatic embryo formation in carnation (Seo *et al.,* 2007).

Protocols for indirect somatic embryo formation have been reported for a limited number of cultivars (Frey *et al.*, 1992). However, in all mentioned cases the regeneration efficiency is quite low. Conditions efficient for direct somatic embryogenesis and plant regeneration were first reported from leaf explants cultured in MS liquid medium (Yantcheva *et al.*, 1998). Direct somatic embryogenesis from leaf explants of *D. caryophyllus* along with *D. barbatus* and *D. chinensis* has also been developed (Pareek and Kothari, 2003). Secondary embryogenesis in carnation has also been reported using petals as explant by Karami *et al.* (2007).

Dianthus is usually vegetatively propagated so it is infected with one or more virus, which subsequently reduces its vigour as well as the size of its flower (Danial *et al*., 2009). Meristem culture of *D. caryophyllus* is reported where the obtained virus-free regenerated plants were even acclimatized to the greenhouse environment (Gutierrez-Miceli *et al*., 2010).

Protoplast culture is important for somaclonal variation, somatic hybridization and direct or vector mediated gene transfer. Regeneration from the protoplast culture of seventeen cultivars of *Dianthus* including *D. caryophyllus, D. chinensis,* and *D. barbatus* has been described using leaves of axenic shoot cultures as explants (Nakano and Mii, 1992; Kim and Lee, 1996). Somatic hybridisation has also been reported between *D. chinensis* and *D. barbatus* (Nakano and Mii, 1993 b).

Increased shoot and root induction using high $AgNO_3$ concentrations were reported for *Dianthus* (Gutiérrez-Miceli *et al*., 2010), *Triticum* and *Nicotiana* (Purnhauser *et al*., 1987) and *Brassica napus* (De Block *et al*., 1989). An additive effect of heavy metals in stimulating morphogenesis in callus redifferentiation has been reported using Kinetin and $AgNo_3$ in *D. caryophyllus*

(Gutiérrez-Miceli *et al.*, 2010) and also cobalt (Roustan *et al*., 1989; Sethi *et al*., 1990; Chraibi *et al*., 1991) and nickel (Roustan *et al*., 1989).

In vitro carnation culture faces the problem of 'hyperhydricity' (Casas *et al*., 2010). Hyperhydricity during micropropagation of carnation was reduced to 0% by media modification. Increased concentration of iron and/or magnesium reduced hyperhydricity with 0.7-0.8% agar and increased shoot multiplication (Yadav *et al*., 2003**).**

Numerous reports on *Agrobacterium tumefaciens* mediated transformation using petal and leaf explant (Van Altvorst *et al.*, 1996); shoot explants (Kinouchi *et al.,* 2006); nodal explants (Nontaswatsri and Fukai, 2010) have been reported.

RAPD analysis is a quick and economic technique to identify genetic similarity among genotypes. The pattern of amplified bands obtained by RAPD could be used for clonal fidelity (Welsh and McClelland, 1990). RAPD based approach is capable of describing the qualitative and quantitative characteristics of the tissue culture induced variation.

RAPD markers have been utilized for screening the clonal fidelity of somatic embryogenesis-derived regenerants of oil palm (Rival *et al.*, 1998), *in vitro* raised bulbets of *Lilium spp.* (Varshney *et al.*, 2001), micropropagated pineapple (Feuser *et al.,* 2003), shoots buds of *Robinia pseudoacacia* (Carvalho *et al.,* 2004), micropropagated teak (Gangopadhyay *et al.,* 2004), adventitious bud from leaf and shoot tips of two micropropagated *Drosera* species (Kawiak and Lojkowska, 2004), long-term micropropagated shoots of banana (Lakshmanan *et al.,* 2007), clonally propagated axillary bud and shoot apices of a clonal rootstock of apple (Gupta *et al.,* 2009) and regenerated shoots of *Capparis deciduas* (Tyagi *et al.*, 2010).

METHODOLOGY

PLANT MATERIAL

Dianthus, an important horticultural plant will be taken for the present study. The seeds of *Dianthus* (L.) would be procured from Namdhari Seed Agency, Bengaluru (India).

CLASSIFICATION

Kingdom	:	Vegetabile
Sub-Kingdom	:	Angiospermae
Division	:	Dicotyledonae
Class	:	Polypetalae
Order	:	Caryophyllinae
Family	:	Caryophyllaceae
Genus	:	*Dianthus*

DESCRIPTION OF THE PLANT

Dianthus belongs to the family Caryophyllaceae which encompasses certain ornamentally important plants. The family comprises of about 75 genera and 2,000 species of dicotyledonous plants out of which mostly are herbs and rarely sub-shrubs.

It is an herbaceous perennial plant growing to 80 cm tall. The leaves are glaucous greyish green to blue-green, slender, up to 15 cm long. The flowers are produced singly or in group of five together in a cyme, and sweetly scented. The original natural flower colour is bright pinkish-purple, but cultivars of other colours, including red, white, yellow and green have been developed.

Various explants from aseptically germinated seeds would be taken for the present study.

Explants to be taken from aseptically grown seedlings:

1. Hypocotyl (0.5-0.7 cm)
2. Cotyledon (whole expanded lamina along with petiole 1-2 mm)
3. Cotyledonary node with shoot tip and part of hypocotyls (1-2 mm) after separating both the cotyledons

Explants to be taken from aseptically grown mature plants:

1. Leaf
2. Nodes
3. Internodes
4. Shoot tips

STERILIZATION AND PREPARATION OF EXPLANTS

The seeds would be washed with 20% (v/v) Extran followed by four rinses in distilled water. Seeds would be surface sterilized in 0.1% (w/v) mercuric chloride ($HgCl_2$) aqueous solution for 3 minutes with continuous stirring and washed thoroughly with three changes of sterile distilled water to remove any traces of $HgCl_2$ in the Laminar Air Flow cabinet.

The seeds would be then germinated on half strength MS media with 3% (w/v) sucrose and solidified with 0.8% (w/v) agar, pH adjusted to 5.8. Explants as stated above would be excised from respective donor plants. These explants would be inoculated on MS medium supplemented with varying concentrations of cytokinins added singly or in combination with different auxins to standardize the protocol for *Dianthus* micropropagation.

BASAL MEDIUM PREPARATION (CONTROL)

Basal medium used in the study would be MS medium. Stock solutions of various inorganic and organic nutrients would be prepared at various concentrations by dissolving their weighed amount in distilled water. For preparing the stock solution of growth regulators; auxin would be dissolved in 2-3 drops of ethyl alcohol and cytokinins in 2-3 drops of 1N HCl. The final volume would be made up by adding distilled water.

The medium would be prepared in a 1000 ml measuring cylinder with distilled water and all the mineral nutrients and sugar (dissolved in small volume of water) would be added. The medium would be made up to the final volume by adding distilled water. The pH of the medium would be adjusted to 5.8 by using HCl (0.01, 0.1, 1N) and NaOH (0.01, 0.1, 1N).

Weighed amount of agar would be added to the medium and dissolved by placing the beaker on a hot plate. Finally it would then be dispensed into 100 ml pre-marked flasks, plugged with non-absorbent cotton and autoclaved at 121^0C and 1.06-kg/cm^2 pressure for 20 minutes.

PROTOCOL FOR *DIANTHUS* CULTURES

The excised explants from the aseptically grown plantlets would be inoculated on MS medium supplemented with cytokinins. The shoot buds would be induced after approximately 4 weeks of culture. The primary shoots induced from the explant would be transferred to MS medium fortified with cytokinins as well as auxins in different concentration. Plantlets with well developed root and shoot system would be transplanted to earthen pots and transferred to green house conditions followed by field transfer.

PREPARATION OF MEDIUM WITH VARIED CONCENTRATION OF HEAVY METALS

The basal medium would be prepared as mentioned above and distributed in beakers and then the different concentrations of heavy metals would be added into separate beakers. The beakers would be marked accordingly. The volume of the medium in each beaker would be raised to the final volume required by adding distilled water and dispensed into culture flasks.

CLONAL FIDELITY ASSESSMENT USING RAPD TECHNIQUE

DNA would be extracted from the tissue culture-derived plantlets. The extracted DNA would be subjected to amplification using PCR technique. RAPD analysis using arbitrary universal

oligonucleotide primers would be employed in order to investigate the genetic fidelity of plant tissue culture-derived regenerants of *Dianthus*.

BIBLIOGRAPHY

Ali, A., H. Afrasiab, S. Naz, M. Rauf and J. Iqbal (2008) An efficient protocol for *in vitro* propagation of carnation (*Dianthus caryophyllus*) *Pak. J. Bot.* **40(1)**: 111-121

Aminuddin, S. K. R., K. M. Srivastava and B. P. Singh (2007) Natural infection of cucumber mosaic virus on *Dianthus barbatus* in India. *Plant Pathol*. **42(5)**: 811-813

Baker, R. and D. J. Philips (1962) Obtaining pathogen free stock by shoot tip culture. *Phytopathol*. **52**: 1242-1244

Bird, R. (1994) Border Pinks pp. 1-174 *Timber Press*, Portland, Oregon, USA.

Carvalho, L. C., L. Goulao, C. Oliveira, J. C. Goncalves and S. Amancio (2004) RAPD assessment for identification of clonal identity and genetic stability of *in vitro* propagated chestnut hybrids. *Plant Cell Tiss. Org. Cult.* **77**: 23-27

Casas, J. L., E. Olmos and A. Piqueras (2010) *In vitro* propagation of carnation (*Dianthus caryophyllus*) pp. 109-110 In: *Protocols for In vitro Propagation of Ornamental Plants, Methods in Molecular Biology* (Edited by: S. M. Jain and S. J. Ochatt) Humana Press.

Choudhary, M. L. and C. K. Chin (1995) Somatic embryogenesis in cell suspension culture of carnation (*Dianthus caryophyllus* L.) *Plant Growth Regulation* **16**: 1-4

Chraibi, K. M., A. Latche, J. P. Roustan and J. Fallot (1991) Stimulation of shoot regeneration from cotyledons of *Helianthus annus* by ethylene inhibitors, silver and cobalt. *Plant Cell Rep.***10**: 204-207

Crouch, N. R. and J. Van Staden (1993) *In vitro* culture of *Dianthus zeyheri subsp.* Natalensis, a south African carnation. *Plant Cell Tiss. Org. Cult.* **35**: 81-85

Danial, G. H., A. N. Yousif and M. S. Omar (2009) Clonal propagation of *Dianthus caryophyllus* L. through tissue culture. *J. Duhok. Univ.* **12(1)**: 91-95

De Block, M., D. De Brouwer and P. Tennig (1989) Transformation of *Brassica napus* and *Brassica oleracea* using *Agrobacrium tumefaciens* and the expression of the *bar* and *neo* genes in the transgenic plants. *Plant Physiol.* **91**: 694-701

Demmink, J. F., J. B. M. Clusters and J. H. W. Bergervoet (1987) Gynogenesis to by pass crossing barriers between diploid and tetraploid *Dianthus species*. *Acta horticulture*. **212**: 167-176

Duhoky, M. M. S., M. A. Salman and M. E. M. Amin (2009) Micropropagation of carnation (*Dianthus caryophyllus*) *J. Duhok Univ.* **12(1)**: 61-66

Earle, E. D. and R. W. Langhans (1975) Carnation propagation from shoot tips cultured in liquid medium. *Hort. Science*. **10**: 608- 610

Evans, D. A., W. R. Sharp and C. E. Flick (1981) Growth and behaviour of cell cultures: embryogenesis and organogenesis pp. 45-113 In: *Plant Tissue Culture* (Eds.) T. A. Thorpe. Academic Press. New York.

Feuser, S., K. Meler, M. Daquinta, M. P. Guerral and R. O. Nodari (2003) Genotypic fidelity of micropropagated pineapple (*Ananas comosus*) plantlets assessed by isozymes and RAPD markers. *Plant Cell Tiss. Org. Cult.* **72**: 221-227

Fisher, M., M. Ziv and A. Vanstein (1993) An efficient method for adventitious shoot regeneration from cultured carnation petals. *Sci Hort.* **53**: 231-237

Frey, L., Y. Saranga and J. Janick (1992) Somatic embryogenesis in carnation. *Hort. Science.* **27**: 63-65

Galbally, J. and E. Galbally (1997) Carnations and pinks for garden and greenhouse. 1-130. *Timber Press*, Portland, Oregon, USA.
Gangopadhyay, G., S. B. Gangopadhyay, R. Poddar, S. Gupta and K. K. Mukherjee (2004) Micropropagation of *Tectona grandis*: Assessment of genetic fidelity. *Biol. Planta*. **46**: 459-461
Ghosh, S. (1987) Micropropagation of Sim's carnation (*Dianthus caryophyllus* L.) *Indian J. Exp. Biol.* **24**: 703-704
Gupta, R., M. Modgil and S. K. Chakrabarti (2009) Assessment of genetic fidelity of micropropagated apple rootstock plants, EMLA 111, using RAPD markers. *Ind J. Exp. Biol.* **47**: 925-928
Gutierrez-Miceli, F. A., L. Arias, N. Juarez-Rodriguez, M. Abud-Archilla, A. Amaro-Reyes and L. Dendooven (2010) Optimization of growth regulators and silver nitrate for micropropagation of *Dianthus caryophyllus* L. with the aid of a response surface experimental design. *In Vitro Cell Dev. Biol. - Plant* **46**: 57-63
Hussain, I., A. Mushtaq and A. Qureshi (1994) *In vitro* multiplication of *Dianthus caryophyllus* L. cv. CSU. Pink and White Sim. *Sarhad J. Agri*. **10**: 539-546
Illahi, I., A. Fareena and J. Mussarat (1995) Tissue culture studies for micropropagation of carnation (*Dianthus caryophyllus* L.). *Pak. J. Bot*. **27**: 411-415
Jethwani, V. and S. L. Kothari (1993) Micropropagation of *D.barbatus* and *D. chinensis* through cotyledonary node culture. *Plant Cell Tiss. Org. Cult.* **3**: 91-96
Jethwani, V., Sharma V. and S. L. Kothari (1994) Micropropagation of *Dianthus chinensis* and *D. barbatus* through shoot tip culture. *J. Bot. Society* **73**: 357-358
Jethwani, V. and S. L. Kothari (1996) Phenylacetic acid induced organogenesis in culture leaf segments of *Dianthus chinensis*. *Plant Cell Rep*. **5**: 869-872
Kallack, H., I. Hilpus and K. Virmuae (1996) Influence of genotype and growth regulations on morphogenetic process in carnation shoot apex cultures. *Sci. Hort.* **65**: 181-189
Kantia, A. and S. L. Kothari (2002) High efficiency adventitious shoot bud formation and plant regeneration from leaf explants of *Dianthus chinensis* L. *Sci. Hort.* **96**: 205-212
Karami, O., Delijou A. and Kordestani G. K. (2007) Secondary somatic embryogenesis of carnation (*Dianthus caryophyllus* L.) *Plant Cell Tiss. Org. Cult.* **92**: 273-280
Karami, O. (2008) Induction of embryogenic callus and plant regeneration in carnation (*Dianthus caryophyllus* L.) *Online J. Biol. Sci.* **8(4)**: 68-72
Kawiak, A. and E. Lojkowska (2004) Application of RAPD in the determination of genetic fidelity in micropropagated *Drosera* plantlets. *In vitro Cell Dev. Biol.-Plant.* **40**: 592-595
Kim J. C. and E. A. Lee (1996) Plant regeneration from mesophyll protoplast of *Dianthus superbus*. *Plant Cell Reports* **16**: 18-21
Kinouchi, T., R. Endo, A. Yamashita and S. Satoh (2006) Transformation of carnation with genes related to ethylene production and perception: towards generation of potted carnations with a longer display time. *Plant Cell Tiss. Org. Cult.* **86**: 27-35
Lakshmanan, V., S. R. Venkataramareddy and B. Neelwarne (2007) Molecular analysis of genetic stability in of long-term micropropagated shoots of banana using RAPD and ISSR markers. *Electro. J. Biotech.* **10.**
Leshem, B., E. Werker And D. P. Shalev (1988) The effect of cytokinins on vitrification in melon and carnation. *Ann. Bot*. **62**: 271-276
Messeguer, J., M. C. Arconada and E. Mele (1993) Adventitious shoot regeneration of carnation (*Dianthus caryophyllus* L.) *Sci. Hort.* **54**: 153-163

Miller, R. M., V. Kaul, J. F. Hutchinson, G. Maheswaran and D. Richards (1991) Shoot regeneration from fragmented flower buds of carnation (*Dianthus caryophyllus* L.) from axillary bud explant. *Ann. Bot.* **67**: 35-42

Moyal-Ben Zvi, M. and A. Vanstein (2007) Carnation. *Biotechnology in Agriculture and Forestry*. Transgenic Crops VI (edn by E. C. Puz and M. R. Davey) **61**.

Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**: 473-497

Nakano, M. and M. Mii (1992) Protoplasts culture and plant regeneration of several species in the genus *Dianthus*. *Plant Cell Rep*. **11**: 225-228

Nakano, M. and M. Mii (1993 a) Antibiotics stimulate somatic embryogenesis without plant growth regulators in several *Dianthus* cultivars. *J. Plant Physiol*. **141**: 721-725

Nakano, M. and M. Mii (1993 b) Somatic hybridisation between *Dianthus chinensis* and *D. babrbatus* through protoplast fusion. *Theo. App. Gen*. **86**: 1-5

Nakano, M., Y. Hashino and M. Mii (1994) Adventitious shoot regeneration from cultured petal explants of carnation. *Plant Cell Tiss. Org. Cult.* **36**: 15-19

Nontaswatsri, C. and S. Fukai (2005) Regenerative callus of *Dianthus* 'Telstar Scarlet' showing mixploidy produce diploid plants. *Plant Cell Tiss. Org. Cult.* **83**: 351-355

Nontaswatsri, C., S. Ruamrungsri and S. Fukai (2009) Callus induction and plant regeneration of *Dianthus chinensis* L. and *Dianthus barbatus* L. via anther culture. *International Society for Horticultural Science*.

Nontaswatsri, C. and S. Fukai (2010) Genetic transformation of Carnation (*Dianthus caryophyllus*) p. 87. In: *Protocols for In vitro Propagation of Ornamental Plants, Methods in Molecular Biology* (Eds: S. M. Jain and S. J. Ochatt) Humana Press.

Nugent, G., T. Wardley and C. Lu (1991) Plant regeneration from stem and petal of carnation (*Dianthus caryophyllus*) *Plant Cell Rep.* **10**: 477-453

Pareek, A. and S. L. Kothari (2003) Direct somatic embryogenesis and plant regeneration from leaf cultures of ornamental species of *Dianthus*. *Sci. Horti.*

Pareek, A., A. Kantia and S. L. Kothari (2004) *In vitro* cloning of ornamental species of *Dianthus. Ind. J. Biotech*. **3**: 363-266.

Petru, E. and Z. Landa (1974) Organogenesis in isolated carnation plant callus tissue cultivated *in vitro. Biol. Planta.* **16**: 450-453

Purnhauser, L., P. Medgyesy, M. Czako, P. J. Dix and L. Marton (1987) Stimulation of shoot regeneration in wheat (*Triticum aestivum*) callus cultures by copper. *Cereal Res. Comm.* **19**: 419-423

Rival, A., L. Bertrand, T. Beule, M. C. Combes, P. Trouslot and P. Lashermes (1998) Stability of RAPD analysis for the detection of somaclonal variants in oil plam (*Elaeis guineensis* Jacq.) *Plant Breed.* **117(1)**: 73-76.

Roustan, J. P., A. Latche and J. Fallot (1989) Stimulation of *Daucus carota* somatic embryogenesis by inhibitors of ethylene synthesis: cobalt and nickel. *Plant Cell Rep.* **6**: 1-4

Sajid, G. M. and S. Pervaiz (2008) Bioreactor mediated growth, culture ventilation, stationary and shake culture effects on *in vitro* growth of sugarcane. *Pak. J. Bot.* **40(5)**: 1949-1956

Sankhla, D., N. Vavis, N. Sanklha and A. Upadhya (1994) *In vitro* regeneration of heat tolerant "German Red" carnation through organogenesis and somatic embryogenesis. *Gartenbouwissenschaft*. **6**: 227-233

Seo, J., S. W. Kim and S. R. Min (2007) High frequency somatic embryogenesis and plant regeneration in root explant cultures of carnation. *Plant Biotecnol. Rep.* **1**: 67-70

Sethi, U., A. Basu and S. Guha-Mukherjee (1990) Control of cell proliferation and differentiation by modulators of ethylene biosynthesis and action in *Brassica* hypocotyl explants. *Plant Sci.* **69**: 225-229

Tyagi, P., S. Khanduja and S. L Kothari (2010) *In vitro* culture of *Capparis decidua* and assessment of clonal fidelity of the regenerated plants. *Biol. Planta.* **54(1)**: 126-130

Van Altvorst, A. C., H. J. J. Koehorst, T. Bruinsma, J. Jansen, J. M. B. Custers, J. De Jong and J. J. M. Dons (1992) Adventitious shoot formation from *in vitro* leaf explant of carnation (*Dianthus caryophyllus* L.) *Sci. Hort.* **51**: 223-235

Van Altvorst, A. C. and H. J. J. Koehorst (1995) Cells within the nodal region of carnation shoots exhibit a high potential for adventitious shoot formation. *Plant Cell Tiss. Org. Cult.* **40**: 151-157

Van Altvorst, A. C., H. J. J. Koehorst, J. D. Jong and J. J. M. Dons (1996) Transgenic carnation plants obtained by *Agrobacterium tumefaciens* mediated transformation of petal explants. *Plant Cell Tiss. Org. Cult.* **45**: 2

Varshney, A., M. Lakshmikumaram, P. S. Srivastava and V. Dhawan (2001) Establishment of genetic fidelity of *in vitro* raised *Lilium* bulblets through RAPD markers. *In vitro Cell Dev. Biol.-Plant.* **37**: 227-231

Villalobos, V. (1981) Floral differentiation in carnation (*Dianthus caryophyllus*) from anthers cultured *in vitro. Phyton.* **41**: 71-75

Welsh, J. and M. McClelland (1990) Fingerprinting genomes using PCR with arbitrary primers. *Nucl. Acids Res.* **8**: 7213-7218

Yadav, M. K., A. K. Gaur and G. K. Garg (2003) Development of suitable protocol to overcome hyperhydricity in carnation during micropropagation. *Plant Cell Tiss. Org. Cult.* **72**: 153-156

Yantcheva, A., M. Vlahova and A. Antanassov (1998) Direct somatic embryogenesis and plant regeneration of carnation (*Dianthus caryophyllus* L.) *Plant Cell Rep* **18**: 148-153